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Modification of lipid phase behavior with membrane-bound cryoprotectants

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Several derivatives of cholesterol containing oxyethylene headgroups with and without a terminal galactose have been synthesized in order to examine the effects of immobilizing a cryoprotectant at a membrane surface. In this work, we have studied the ability of the triethoxycholesterol (TEC) and triethoxycholesterol galactose (TEC-Gal) derivatives to modulate the phase behavior of phosphatidylcholine and phosphatidylethanolamine membranes. Methods of fluorescence polarization, ^{31}P-NMR and freeze-fracture electron microscopy were employed to monitor these changes in lipid phase behavior. Fluorescence polarization data demonstrated the ability of the derivatives to fluidize gel state and rigidify liquid-crystalline state phosphatidylcholines in a manner similar to that observed for cholesterol. Unlike cholesterol, however, the T_m of dipalmitoylphosphatidylcholine (DPPC) was reduced in a concentration-dependent manner with each of the derivatives. Freeze-fracture electron microscopy and ^{31}P-NMR of DOPE dispersions indicate an increase in the lamellar to hexagonal phase-transition temperature on the order of 10–20 C° above room temperature for mixtures with 20 mol% of the derivatives. These results are discussed in terms of the properties exhibited by compounds such as carbohydrates, which are known to serve as cryoprotectants for synthetic and biological membranes.

Introduction

The role of carbohydrate structure and function with respect to membrane stability has been and continues to be an area of intense investigation. In recent years, much discussion has been devoted to the question of interactions of carbohydrate with membrane surfaces. In particular, interactions of carbohydrate with phospholipids have been examined [1–3]. The effects of such interactions include modification of phospholipid phase behavior and stabilization of phospholipid vesicle structure under conditions of freeze-drying and freeze-thawing [4–8].

Cryoprotectants such as dimethyl sulphoxide, glycerol, ethylene glycol and urea have been known and utilized for many years for the cryopreservation of biological materials [9]. Interest in carbohydrates for the same purposes was spurred by the discovery over 10 years ago that certain organisms capable of surviving in a dehydrated state for many years produce large amounts of trehalose, a non-reducing disaccharide of glucose [10,11]. Strauss and co-workers and Crowe et al. have since demonstrated that the addition of trehalose

Abbreviations: TEC, triethoxycholesterol; TEC-Gal, triethoxycholesterol galactose; SUV, small unilamellar vesicles; DPPC, dipalmitoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; TLC, thin-layer chromatography; DPH, 1,3,5-diphenylhexatriene; MLV, multilamellar vesicles; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

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or sucrose to liposomes of synthetic or biological origin enables these structures to retain their morphological and functional characteristics upon freezing and thawing, or dehydration [5–7]. It was found for example, that at concentrations of 0.3 g/g membrane, trehalose preserved the integrity of Ca^{2+} transporting microsomes which had been dehydrated (these structures do not normally survive dehydration) [10]. In terms of effectiveness, trehalose has been shown to be three times more effective than sucrose and several times more effective than other cryoprotectants for these particular membranes [10]. Several postulates have been proposed to explain the mechanism of carbohydrate action. These models are based primarily on evidence demonstrating the ability of carbohydrates to modulate phase behavior [1–8]. The data include observations of the ability of certain carbohydrates to (a) reduce the phase-transition temperature in SUV [4] and (b) to stabilize hexagonal-phase-forming lipids in the lamellar phase [11].

An intriguing question arising from this work is whether carbohydrates directly attached to the membrane surface elicit even more pronounced effects (i.e., modification of phase behavior and stabilization) than carbohydrates free in solution. In such a system, the carbohydrate moiety would be localized at the membrane surface, and so potentially enhance the interactions required for membrane stabilization. Selective modification of a membrane surface could be accomplished with relatively small amounts of the cryoprotectant compared to the concentrations of free carbohydrate required to preserve membrane integrity in dehydrated or frozen systems.

We have prepared the cholesterol derivatives (Fig. 1) for the purpose of examining and optimizing carbohydrate–lipid interactions. The compounds consist of a carbohydrate (galactose) placed at a variable distance from the hydrophobic portion of the molecule via a hydrophilic oxyethylene unit of variable length. These features permit (a) incorporation of the molecule into a bilayer via a steroid anchor, (b) variation in position and mobility of the carbohydrate moiety for maximization of potential interactions and (c) variation in the structure of the carbohydrate. The derivative without the sugar (B) allows direct examination of the requirements for a carbohydrate structure.

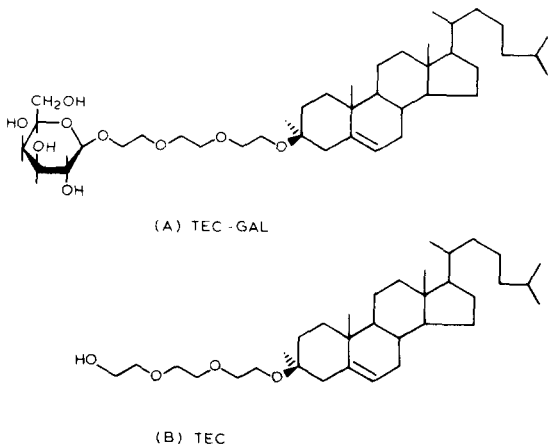


Fig. 1. Structure of cholesterol derivatives. (A) 3,6,9-trioxaoctan-1-β-D-galactosylcholesteryl-3β-ol (TEC-Gal). (B) 3,6,9-trioxaoctan-1-ol-cholesteryl-3β-ol (TEC).

For initial studies, we have examined derivatives where $n = 3$. These compounds are designated as TEC-Gal (1A) and TEC (B). The synthesis is described in the Materials and Methods. The incorporation of these derivatives into phospholipid membranes composed of dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylcholine (DOPC), or dioleoylphosphatidylethanolamine (DOPE) are shown to modify the lipid phase behavior. These effects have been demonstrated to be directly related to the cryoprotective action exhibited by a compound [12]. Several methods have been employed to monitor these alterations.

Fluorescence anisotropy of DPH was utilized to monitor alterations in the membrane ordering and gel to liquid-crystalline phase-transition temperature of DOPC and DPPC respectively. ^{31}P-NMR and freeze-fracture electron microscopy were used to assess changes in the lamellar to hexagonal phase-transition temperature of DOPE. The results are compared to the influence of cholesterol on membrane structure and fluidity. A comparison of the effects of trehalose and other cryoprotectants on these properties is also made.

Materials and Methods

Materials

DOPC, DOPE and DPPC were purchased from

Avanti Polar Lipids, (Birmingham, AL). Cholesterol was purchased from Sigma. 1,3,5-Diphenylhexatriene was purchased from Aldrich.

Methods

Preparation of poly(oxyethylene) derivatives of cholesterol. Samples of the mono-, di-, and triethoxy derivatives of cholesterol were prepared by refluxing the appropriate poly(ethylene glycol) with cholesteryl-*p*-toluenesulfonate in dioxane for 2–3 h [13,14]. The dioxane was removed by rotary evaporation and the resulting material was taken up in water. The milky aqueous layer was extracted with diethyl ether. The organic layer was subsequently washed with a 10% aqueous sodium carbonate solution, dried over anhydrous sodium carbonate, and removed by rotary evaporation. The monoethoxy and diethoxy derivatives yielded solids, whereas the triethoxy derivative formed a liquid-crystal at room temperature. All materials were purified by column chromatography on silica gel, and by recrystallization from methanol for the solids. Samples were characterized by TLC, NMR, mass spectra, and melting point determinations.

Preparation of carbohydrate derivatives. The appropriate cholesterol derivative was dissolved in benzene and placed in a round-bottom flask fitted with an addition funnel. To this was added silver oxide, iodine, and powdered molecular sieves (4 A, dried at 100 °C). Acetobromo-α-D-galactose, which had been dissolved in benzene, was added to the stirred mixture via the addition funnel [15]. The sugar was added dropwise over a period of 1 h. The molar ratio of siver oxide/iodine/sterol/sugar was 2 : 1 : 1 : 2. The mixture was stirred at room temperature in the dark for 3–7 days. The progress was followed by TLC on silica gel. The mixtures were filtered, and the organic solvent was removed by rotary evaporation. The acetate protected glycolipids were purified by column chromatography on silica gel. Samples were characterized by TLC, NMR, mass spectra and melting points.

The acetate groups were removed by dissolving the compounds in methanol to which was added a small quantity of sodium methoxide (0.05 M). The reaction progress was followed by TLC and litmus. The mixture was neutralized at the end of the reaction by treatment with an Amberlite exchange resin. Samples were characterized by TLC, NMR, mass spectra, ultraviolet spectroscopy, and melting points. Specific ($FeCl_3 \cdot 6H_2O$) as well as nonspecific (H_2SO_4) stain reagents were used to develop TLC plates and confirm the presence of sterol and carbohydrate moieties in the glycolipids.

^{31}P-NMR. Samples for ^{31}P-NMR were prepared by drying lipids in 10 mm (o.d.) NMR tubes under nitrogen followed by thorough drying under vacuum for 7–12 h to remove trace solvent. Samples were hydrated with NTE buffer (100 mm NaCl/10 mm Tris-HCl/0.2 mm EDTA (pH 7.4)), and an aliquot of the ^2H_2O analog of this buffer used for signal lock. Lipid concentrations were kept constant at 56 mM. Samples were dispersed by vortex mixing for 10–30 min, or freeze/thawing with vortexing. The duration of vortexing varied according to the phase behavior of the mixture. Samples which formed the hexagonal phase were vortexed for longer periods to assure complete dispersion. In general, samples were prepared at least 1 h prior to use.

All samples were examined using a Bruker 500 MHz NMR with broad-band proton decoupling (for TEC-Gal) or inverse gated decoupling (for TEC). The two decoupling methods did not alter the observed spectra. Free induction decays were collected from 500–1000 transients by employing an 8.0 μs 90° radiofrequency pulse with a 3 s interpulse delay and 50 kHz sweep width. For kinetic studies at various temperature intervals, a 15.0 μs 90° radiofrequency pulse with a 0.04 s interpulse delay was utilized to permit rapid accumulation of spectra. An exponential multiplication corresponding to 100 Hz line-broadening was applied to the free induction decay prior to Fourier transformation.

Fluorescence spectroscopy. Lipid samples were dried down from solvent under a stream of nitrogen and subsequently under vacuum for 6–12 h to remove traces of organic solvent. All samples were hydrated with NTE buffer (100 mM NaCl/10 mM Tris-HCl/0.2 mM EDTA (pH 7.4)) above the phase-transition temperature. The final lipid concentration of each sample was 1.0 μmol/ml. Samples were vortexed for 10 min above the phase-transition temperature. An aliquot of 2 μl of a 2 mM DPH solution (in tetrahydrofuran) was added

to the samples, which were subsequently equilibrated for 2 h above the phase-transition temperature. Values for polarization were collected using an SLM-4800 spectrofluorometer $\lambda_{ex} = 357$ nm, $\lambda_{em} = 430$ nm. A T-format was utilized for placement of the photomultiplier tubes with a long pass filter in the emission channel that cut off light below 430 nm. The temperature in the sample chamber was controlled with a circulating waterbath and monitored using a thermocouple inserted into the cuvette. Samples were stirred continuously during measurement via a magnetic stirrer to prevent the settling of multilamellar vesicles. The effects of scattered light on the measured anisotropies was found to be negligible. Polarization and anisotropy were calculated using the following relationships:

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

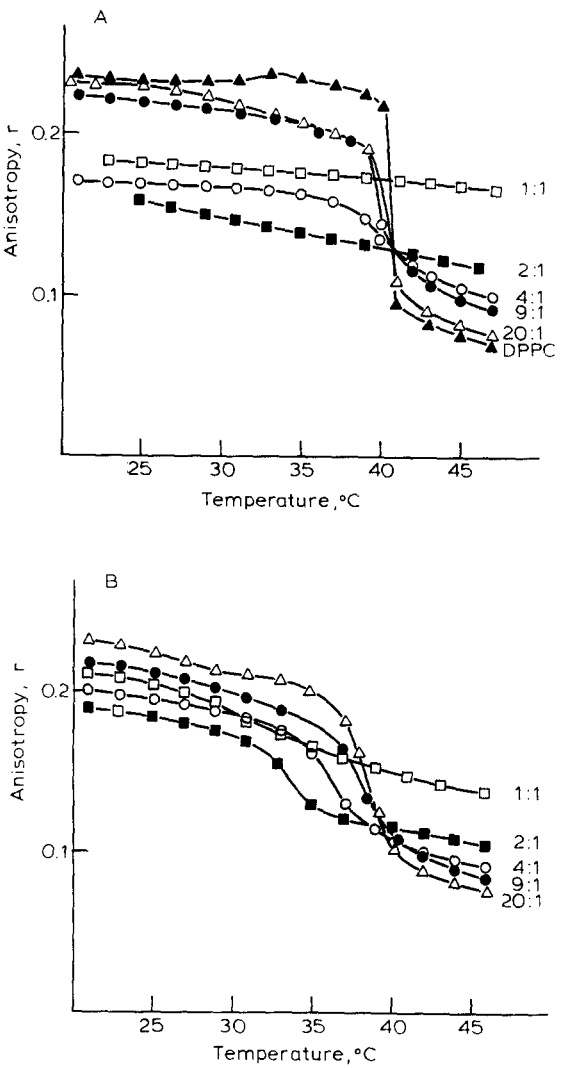
$$r = \frac{2P}{3 - P}$$

Electron microscopy. Samples from ^{31}P-NMR were examined using freeze-fracture electron microscopy. The initial lipid concentration of each was 56.1 mM. Aliquots were spun down for 10–15 s to pellet the lipid. The pellet was then resuspended in a 30% glycerol solution of the buffer of equal volume. Aliquots of these samples were frozen in liquid freon from room temperature and subsequently fractured at −100 °C in a Balzer's apparatus, and etched for 1 min and shadowed with Pt/C. All samples were examined with a Philips EM 201 electron microscope operating at 80 kV.

Results

Fluorescence spectroscopy

Fluorescence anisotropy of DPH is commonly utilized to monitor the gel to liquid crystalline phase transition as well as to estimate membrane fluidity [16–18]. Fig. 2A illustrates the temperature dependence of DPH anisotropy for DPPC MLV with increasing cholesterol content up to 50 mol%. Consistent with previous observation by workers using various techniques (NMR, ESR, fluorescence anisotropy), the addition of cholesterol results in a decrease in the measured anisotropy in the gel state and an increase in the measured anisotropy in the liquid-crystalline state [19–21]. These changes have been associated with a disordering effect of cholesterol on the gel state and an ordering effect on the liquid-crystalline state [22–25]. As the diagram also illustrates, the phase-transition temperature of the lipid in the presence of cholesterol remains relatively constant, although it is broadened to the point of being undetactable at a lipid-cholesterol ratio of 2 : 1.



The behavior of cholesterol may be compared with that obtained for the sterol derivatives (Figs.

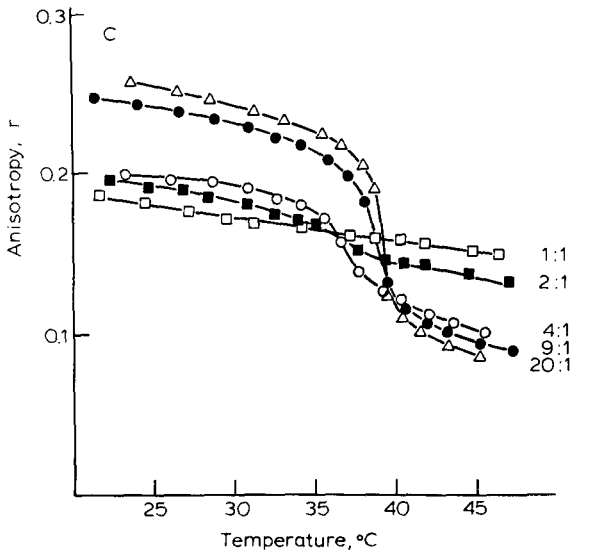


Fig. 2. Anisotropy vs. temperature of (A) DPPC/cholesterol, (B) DPPC/TEC-Gal, (C) DPPC/TEC MLV. ▲ DPPC; △ 20:1; ● 9:1; ○ 4:1; ■ 2:1; □ 1:1.

2B, C). Addition of both TEC and TEC-Gal cause a reduction in the measured anisotropy of DPH in the gel state and an increase in the liquid-crystalline state. The effect is similar to that observed for cholesterol, but is reduced in magnitude. In contrast to cholesterol, both derivatives induce a progressive reduction in the phase-transition temperature with increasing concentration. In both cases, the reduction is linear with concentration and corresponds to a decrease of approx. 0.17–0.20 °C per mol% sterol. Furthermore, the transition is still detected at concentrations of 33 mol% for TEC and 50 mol% TEC-Gal, where no transition is observed for mixtures with cholesterol. Table I summarizes the effects on the phase-transition temperature observed in each case.

To examine further the effect of the derivatives on the ordering of the lipid in the liquid-crystalline state, anisotropy measurements were made on DOPC MLV containing varying proportions of the sterols. DOPC undergoes the gel to liquid-crystalline transition at −22 °C [26]. At room temperature and above it exists in a highly fluid state characterized by low anisotropy values for DPH. With increasing cholesterol, however, the anisotropy increases (Fig. 3A). The magnitude of the increase offers an estimate of the ordering

TABLE I

PHASE-TRANSITION TEMPERATURE AS A FUNCTION OF STEROL CONTENT FOR DPPC/CHOLESTEROL, DPPC/TEC AND DPPC/TEC-Gal

S.D. are in parentheses. Chol, cholesterol.

Mol% sterol	Phase-transition temperature (°C)		
	Chol	TEC	TEC-Gal
0	40.5	40.5	40.5 (0.7)
5	40.3	39.3	39.0 (0.7)
10	39.8	38.8	38.5 (0.7)
20	40.0	37.0	37.2 (0.6)
33	–	35.1	33.6 (0.6)
50	–	–	31.9 (0.4)

effect which cholesterol exerts on the bilayer [27,28]. For TEC and TEC-Gal, similar ordering was observed, although slightly reduced in magnitude (Figs. 3B and C; Table II). These results are consistent with those reported by Lyte and Shinitzky for egg PC/cholesteryl phosphorylcholine mixtures and with Lee's results for DOPC/cholesteryl hemisuccinate mixtures [27,29].

^{31}P-NMR spectroscopy

^{31}P-NMR is frequently employed to study the phase behavior of lipids in natural and artificial systems [30,31]. In particular, ^{31}P-powder patterns offer a convenient way to monitor transitions between lamellar, hexagonal, cubic or micellar phases. For lipids in a lamellar phase, restricted motion of the lipids results in only partial averaging of the large chemical shift anisotropy of the ^{31}P nucleus. The basic features of the spectrum generated under these conditions include a low

TABLE II

ANISOTROPY OF DPH AT 37 °C AS A FUNCTION OF STEROL CONTENT FOR DOPC/CHOLESTEROL, DOPC/TEC AND DPPC/TEC-Gal

Mol% sterol	Anisotropy		
	Chol	TEC	TEC-Gal
0	0.060	0.060	0.060
5	0.063	0.053	0.060
10	0.067	0.056	0.066
20	0.079	0.080	0.081
33	0.106	0.087	0.091
50	0.160	0.120	0.112

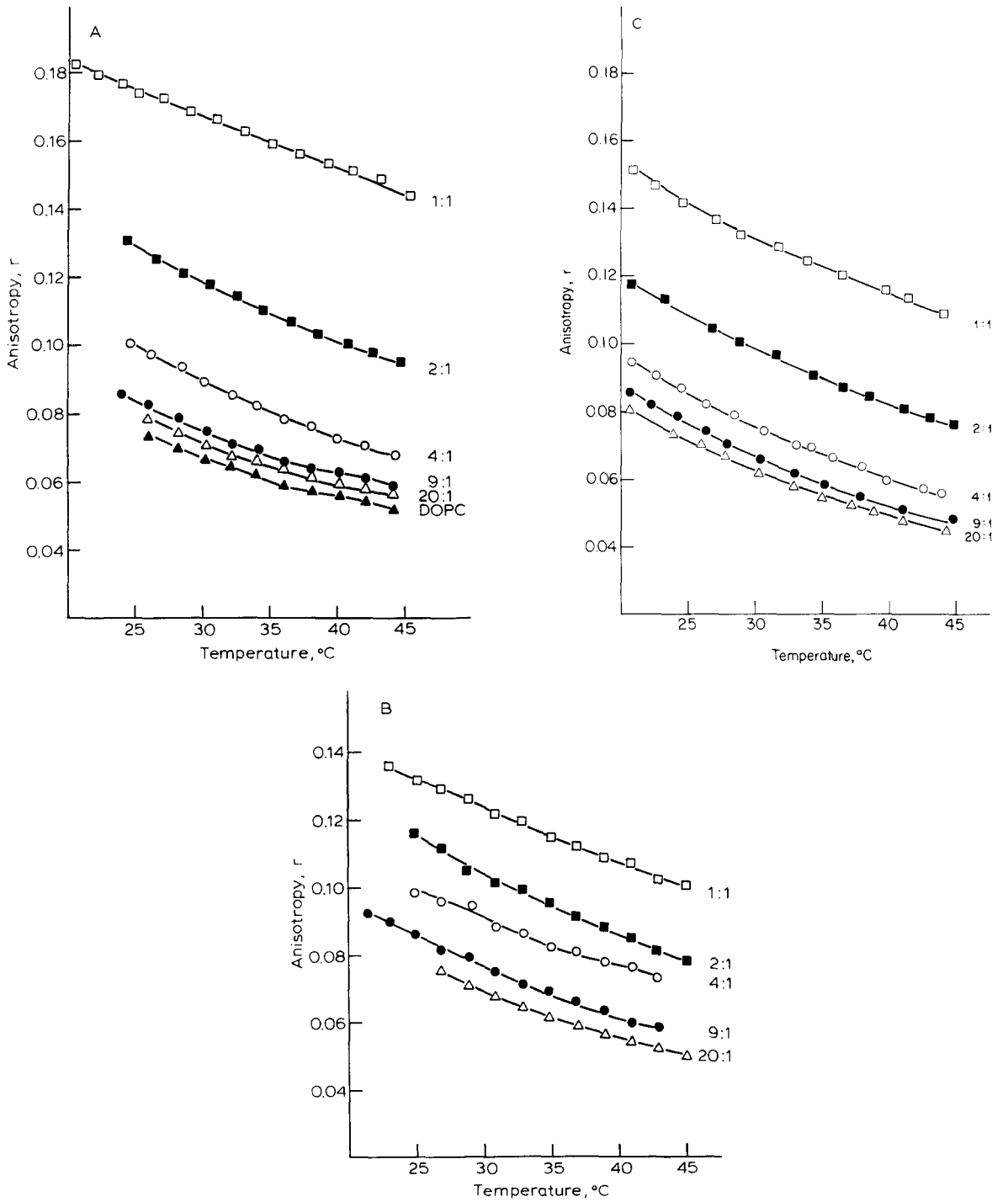


Fig. 3. Anisotropy vs. temperature of (A) DOPC/cholesterol, (B) DOPC/TEC-Gal, (C) DOPC/TEC MLV. ▲ DOPC; △ 20:1; ● 9:1; ○ 4:1; ■ 2:1; □ 1:1.

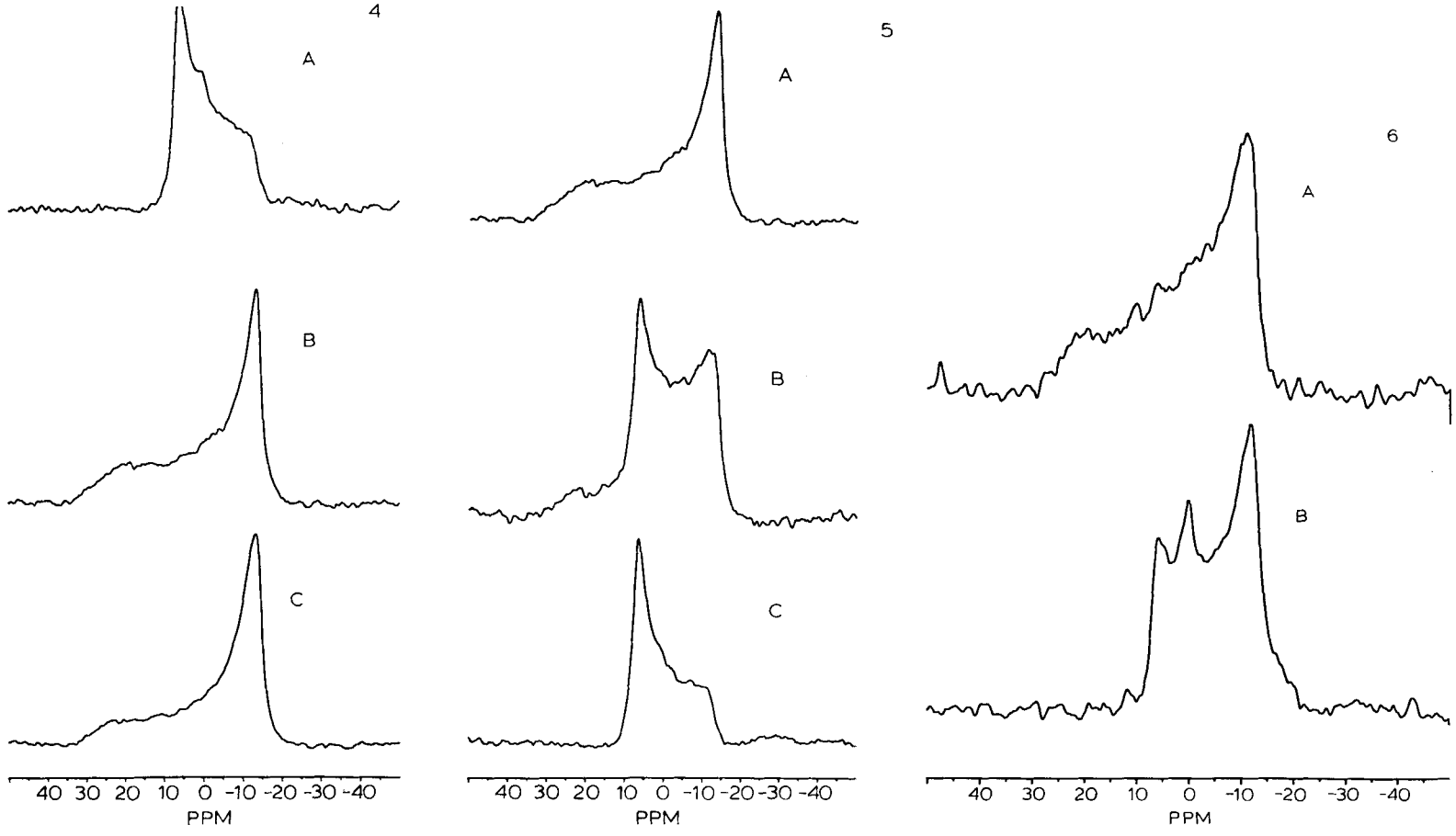


Fig. 4. ^{31}P-NMR powder patterns for DOPE/TEC dispersions at 24 ° C. (A) 10 mol% TEC; (B) 20 mol% TEC; (C) 33 mol% TEC.

Fig. 5. Temperature dependence of ^{31}P-NMR powder patterns for DOPE/TEC (4 : 1) at (A) 24 ° C, (B) 30 ° C and (C) 35 ° C.

Fig. 6. Temperature dependence of powder patterns for DOPE/TEC-Gal (4 : 1) at (A) 45 ° C and (B) 55 ° C.

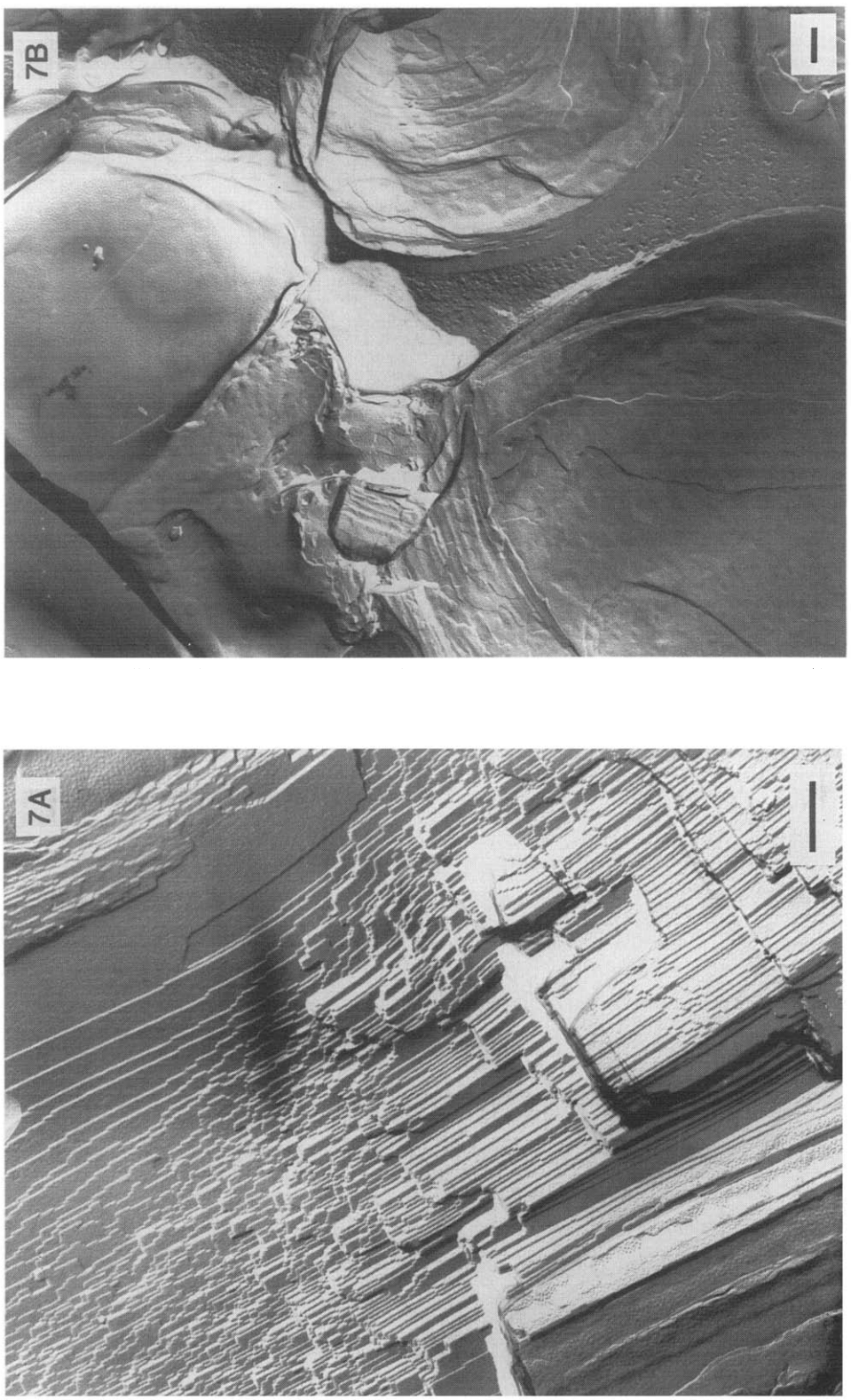


Fig. 7. Freeze-fracture electron micrographs of (A) DOPE/TEC-Gal (9:1) mixture showing presence of hexagonal phase; (B) DOPE/TEC-Gal (9:1) showing presence of lamellar phase. Bar equals 0.24 μm in all panels.

field shoulder and high field peak separated by $\Delta\sigma = -40$ ppm. Lipids in the hexagonal phase can undergo additional motional averaging due to lateral diffusion around the aqueous channels which the tubules enclose. The line-shape generated under such conditions has reversed symmetry in comparison to the lamellar spectra, and is narrowed by a factor of 2. Finally, lipids in vesicles, micelles or phases, such as cubic or rhombic, allow isotropic (complete) averaging of the tensor. This occurs due to diffusional motions of the lipids over a wide variety of orientations on a time scale of at least 10^{-8} cm^2/s [32,33]. Under these conditions, a narrow symmetric ^{31}P-spectrum is generated.

Unsaturated phosphatidylethanolamines normally adopt a hexagonal phase (H_{II}) arrangement at a given temperature and pH [33,34]. The exact temperature at which this transition occurs for a particular type of PE is sensitive to hydration, acyl chain composition, pH and the presence of particular solutes [35–38]. The propensity of PE to adopt the hexagonal phase arrangement may be explained in terms of the fact that PE is normally

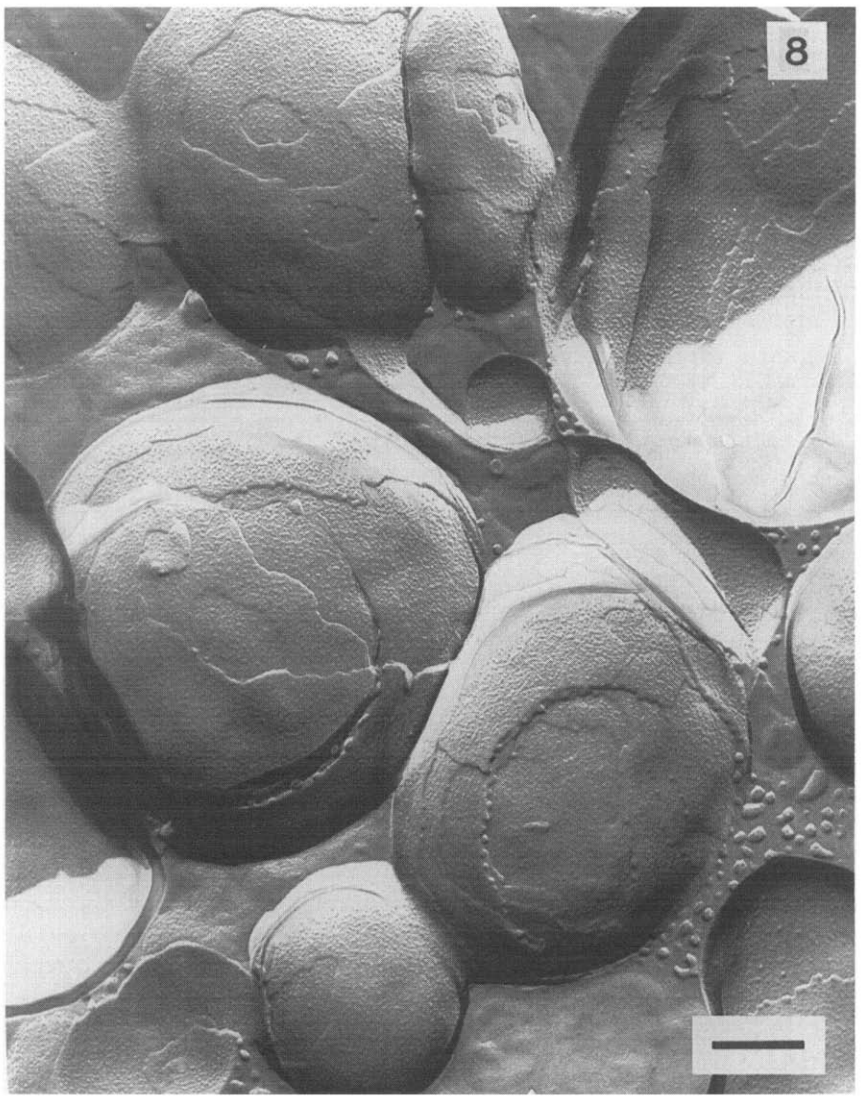


Fig. 8. Freeze-fracture electron micrograph of DOPE/TEC-Gal (4:1). Bar equals 0.24 μm.

poorly hydrated, an effect which is enhanced by the potential for strong hydrogen bonding among the headgroups [35–38].

DOPE undergoes the lamellar to hexagonal transition at approx. 10–18 °C [35]. Incorporation of cholesterol in the membrane promotes the formation of the hexagonal phase. This effect has been demonstrated by ^{31}P-NMR and is attributed to the packing constraints imposed by the steroid due to its relatively small poorly hydrated head group and large hydrophobic body [39].

Fig. 4 illustrates the effect of various mole ratios of TEC and TEC-Gal on the phase behavior of DOPE. In contrast to cholesterol, these derivatives increase the temperature at which the lamellar phase exists. For both TEC and TEC-Gal, the minimum derivative concentration required to stabilize DOPE fully into a lamellar phase at room temperature is 20 mol%. To assess the extent of the stabilization at this composition, the temperature dependence of the spectra was examined. For the DOPE/TEC mixture (4:1) (Fig. 5), DOPE begins to enter the hexagonal phase at approx. 30 °C. The transition is complete by 35 °C. DOPE/TEC-Gal mixtures remain in the lamellar phase at temperatures up to 45 °C. There is little variation in the spectra at this temperature for at least 1 h. Heating above 45 °C, however, induces the formation of hexagonal and isotropic components of the spectra (Fig. 6). Upon cooling the sample back to room temperature, spectra characteristic of the lamellar phase are regenerated. It thus appears that the lamellar phase is the equilibrium phase at room temperature for these compositions.

Electron microscopy results

The ^{31}P-NMR results presented above are corroborated by freeze-fracture electron microscopy. Figs. 7A and B depict samples prepared from a 9:1 mixture of DOPE/TEC-Gal. These samples are characterized by two distinct regions. There are regions in the same sample preparation possessing the regular, ribbed appearance characteristic of hexagonal phase arrangement, and regions with the smooth fracture faces characteristic of the lamellar phase in the form of MLV [40,41]. These results are consistent with those obtained from ^{31}P-NMR, where the presence of hexagonal and lamellar components in the spectra were detected.

Fig. 8 depicts the freeze-fracture morphology of samples of DOPE/TEC-Gal (4:1) mixtures. The structures are characteristic of MLV of 0.2–2.0 μm in diameter. This is again consistent with the ^{31}P-NMR results which indicated the presence of the lamellar phase alone for this composition.

Discussion

The results appearing in the previous section demonstrate the capacity of the derivatives to alter the phase behavior of phospholipids. For MLV composed of DPPC, a reduction in the phase transition temperature is observed similar to the 7 °C depression in T_m for PC/PS (9:1) SUV upon addition of 1 M trehalose [4]. The implication of this is that the lateral spacing between the molecules in the bilayer is increased. This lowering of the T_m is opposite to the trend reported by Crowe et al. for MLV in the presence of trehalose [4]. These authors indicate that the increase in T_m for the MLV may be a consequence of only a few of the outer lamellae being exposed to the trehalose, resulting in dehydration of the inner lamellae. It is anticipated that equal distribution of the carbohydrate throughout the lamellae would produce a behavior similar to the PC/PS SUV and the DPPC MLV used in this study, since it is incorporated into the lamellae.

In addition to the effects of trehalose on PC, it is also known to inhibit the formation of the hexagonal phase arrangement for DOPE [11]. The bilayer to hexagonal phase transition is shifted by as much as 20–30 °C above the normal temperature in the presence of trehalose (4 mol/mol lipid). A similar behavior is observed here for the cholesterol derivatives. For TEC-Gal, the lamellar to hexagonal phase-transition temperature is shifted to a temperatures approx. 30 °C higher than that for pure DOPE. An increase in the lamellar to hexagonal phase-transition temperature is also observed for 20 mol% TEC, but the shift is less dramatic.

Several mechanisms may be invoked to explain the results obtained in the case noted above. These include the possibilities that: (i) the derivatives alter the long-range order of water molecules or

hydration capacity of the membrane surface. This would result in alterations of phospholipid headgroup orientation and interactions [42]; (ii) some type of coordinate linkage may occur between the derivatives and the phospholipid headgroups such as hydrogen bonding [43]; (iii) the derivatives may interact with the hydrocarbon chains via the steroid portion of the molecule [44]; (iv) steric contraints imposed by the derivatives may alter lipid packing and interactions [45].

All four mechanisms offer tenable explanations for the effects of the derivatives on phosphatidylcholine and phosphatidylethanolamine. For DPPC, the consequence of each of these would be an increase in the lateral spacing of the phospholipids, effectively decreasing the Van der Waals interactions and lowering the T_m as observed [42,43]. For DOPE, an increased spacing and hydration would decrease the potential for hydrogen bonding among the headgroups, which is partially responsible for formation of the hexagonal (H_{II}) phase. Disruption of the intermolecular hydrogen bonding in this manner would promote an entropic driving force for stabilization into the lamellar phase. While present data do not implicate any one of these in the modulation of lipid phase behavior, future studies should elucidate the underlying mechanisms involved.

In summary, the derivatives demonstrate the capacity to lower the gel to liquid-crystalline phase-transition temperature in PC and inhibit the formation of the H_{II} phase for PE. Both appear to be properties of agents used as cryoprotectants. The majority of cryoprotectants studied to date possess this feature [12]. The effects elicited by the derivatives are more pronounced on a concentration basis than that observed for free carbohydrates. Based on the calculated concentration of the cryoprotectants within a fixed distance from the membrane surface, however, the effects are of the same order of magnitude. This implies that immobilization of a cryoprotectant at the membrane surface is a viable strategy for reducing the amounts of material required to modulate phase behavior. An intriguing question posed by these results is whether or not this approach is equally effective on the property of cryoprotection. The direct structural requirements (such as the presence or absence of carbohydrate, its mobility and position, etc.) needed for producing the cryoprotective action for this system remain to be assessed.

Acknowledgements

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